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Journal of Cereal Science 43 (2006) 31–37

Journal of
CEREAL
SCIENCE

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Phosphomannose isomerase, *pmi*, as a selectable marker gene for durum wheat transformation

A. Gadaleta[a,*], A. Giancaspro[a], A. Blechl[b], A. Blanco[a]

[a]*Genetics and Plant Breeding Section, Department of Environmental and Agro-Forestry Biology and Chemistry, University of Bari, Via Amendola 165/A, 70126 Bari, Italy*
[b]*USDA-ARS Western Regional Research Center, Albany, CA 94710-1105, USA*

Received 18 January 2005; revised 8 June 2005; accepted 16 June 2005

Abstract

Transgene technology provides a powerful tool for developing traits that are otherwise difficult to achieve through conventional methods. The development of transgenic plants requires the use of selectable marker genes, as the efficiency of plant transformation is less than optimal for many important species, especially for monocots such as durum wheat (*Triticum turgidum* var. *durum*). Many concerns have been expressed about the persistence of currently used marker genes in plants used for field cultivation. To sustain further progress in this area, alternative efficient selection methods are desirable. A recent development is the use of selective genes that give transformed cells a metabolic advantage (positive selection) compared to untransformed cells, which are slowly starved with a concomitant reduction in growth and viability. This selection strategy is in contrast to traditional negative selections during which the transgenic cells are able to survive on a selective medium whereas the non-transgenic cells are actively killed by the selective agent. We compared the 'selection efficiency' of a commonly used negative selection method that employs the *Streptomyces hygroscopicus bar* gene to confer resistance to the herbicide bialaphos, to a positive selection employing the *Escherichia coli* phosphomannose isomerase (*pmi*) gene as the selectable gene and mannose as the selective agent. Calli derived from immature embryos of the durum wheat cultivar Svevo were bombarded separately with *bar* and *pmi* genes using a biolistic system. The integration and expression of the two genes in the T_0 generation were confirmed by PCR analysis with specific primers for each gene and the chlorophenol red assay, respectively. The selection efficiency, calculated as the number of expressing plants divided by the number of total regenerants, was higher when *pmi* was used as the selectable marker gene (90.1%) than when *bar* was used (26.4%). Thus, an efficient selection method for durum wheat transformation was established that obviates the use of herbicide résistance genes.

Keywords: *pmi*; *bar*; *Triticum turgidum*; Marker gene

1. Introduction

The development of novel biotechnologies for direct gene transfer has accelerated plant improvement programs, especially for monocotyledonous species such as wheat which have been traditionally recalcitrant to transformation. The process of plant improvement via direct gene transfer of alien genes involves the insertion of well-characterized genes into regenerable embryogenic cells and subsequent recovery of fully fertile plants with the inserted gene integrated into their genomes. These novel technologies allow access to an unlimited gene pool for genetic enrichment of crop plants without the constraint of sexual compatibility. Incorporation of genes for insect, disease, or herbicide resistance into crop plants has either increased their productivity or decreased the costs of production (Jauhar, 2001).

Despite the recent advances in the transformation of plants, technologies for the efficient identification of transgenic plants in the absence of any selection have been optimized for only a few species such as tobacco (Hohn et al., 2001). Identification of transgenic plants in

Abbreviations: CPR, chlorophenol-red assay; MS, Murashige and Skoog; PCR, polymerase chain reaction.

* Corresponding author. Tel.: +39 080 544 2526; fax: +39 080 544 2200.
E-mail address: agata.gadaleta@agr.uniba.it (A. Gadaleta).

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doi:10.1016/j.jcs.2005.06.004

the important crop species, which have relatively low transformation efficiencies, requires the use of selectable marker genes to minimize regeneration of non-transformed plants (Hohn et al., 2001). The most frequently used selectable markers are genes conferring resistance to herbicides or antibiotics. These include *bar* and *pat* genes conferring resistance to phosphinothricin, the active ingredient in BASTA and bialaphos herbicides, *EPSPS* (*5-enolpyruvylshikimate-3-phosphate synthase*) conferring resistance to the herbicide glyphosate, and the *bla*, *nptII*, *hph*, *aadA*, and *cat* genes, that encode resistances to the antibiotics ampicillin, kanamycin, hygromycin, spectinomycin and cloramphenicol, respectively. However, the presence of these genes is undesirable in crop plants grown in the field because of concerns for potential harm to environmental and human health. These include the possibility that a herbicide resistance transgene could move from a cultivated plant to its wild relatives, producing a 'super weed' that could be hard to control by the use of available herbicides, and the possibility that antibiotic resistance genes could make their way to bacteria in the environment or human gut, rendering those antibiotics less useful. The first concern could be addressed by the new method of chloroplast transformation, wherein the herbicide resistance is engineered into a crop's chloroplast genome, but this has been successful only in tobacco thus far (Daniell et al., 1998). Another way to mitigate these concerns is to eliminate marker genes after transformants have been identified, using site-specific recombination, which has been successful in some species (Ow, 1996). A third way is to use selections that are not based on toxic compounds. These include genes that allow transformed cells to metabolise sugars such as ribitol (Lafayette and Parrott, 2001) or mannose (Joersbo et al., 1998) which cannot be used by untransformed plant cells. Amino acids have been used as negative and positive selective agents in conjunction with the marker gene, *doa1*, which encodes D-amino oxidase (Erikson et al., 2004). This enzyme catalyses the oxidative deamination of a range of D-amino acids and selection is based on differences in the toxicity of different D-amino acids to plants. An alternative to the use of herbicides or antibiotics as selectable markers is the *cah* (cyanamide hydratase) gene for wheat transformation. The *cah* gene gives the transgenic plant the ability to convert cyanamide into urea as a fertilizer source (Weeks, 2000).

In the present study we compared the 'selection efficiency' of a negative selection method in durum wheat (*Triticun turgidum* var. *durum*) using the *bar* gene to confer resistance to the herbicide bialaphos, to a positive selection based on the *phosphomannose isomerase (pmi)* gene as the selectable gene and mannose as the selective agent. Phosphomannose isomerase catalyzes the reversible interconversion of mannose-6-phosphate and fructose-6-phosphate, allowing plant cells to utilize mannose as a carbon source and to grow and differentiate on media containing mannose (Joersbo et al., 1998).

2. Materials and methods

2.1. Embryo isolation and culture initiation medium

Immature seeds 15–18 days post-anthesis from durum wheat cv. Svevo were surface-sterilized with 70% ethanol for 5 min and 20% sodium hypochlorite for 15 min, then rinsed in sterile water. Immature embryos that varied in size between 0.8 and 1.5 mm in diameter were aseptically excised under a stereo dissecting microscope and placed with the scutellar portion of the embryo exposed on a solid MS medium. The basal callus induction medium, MS, contained the inorganic components of Murashige and Skoog (1962), 150 mg/l L-aspargine, 0.50 mg/l thiamine-HCl, 40 g/l maltose, and 3.5 g/l Phytagel (Sigma, St Louis). Callus induction and maintenance were conducted on media supplemented with 1 mg/l 2,4-D.

2.2. Transformation procedure and plasmid DNA

Only embryogenic calli were used for transformation. Calli were transformed with the pNOV2820 plasmid (provided by Syngenta, Research Triangle Park, North Carolina), containing the *E. coli pmi* gene encoding phosphomannose isomerase, or with pAHC25, containing the selectable *bar* gene under the control of the maize *Ubi1* promoter and first intron (Christensen and Quail, 1996). Bombardments were performed using a PDS 1000\He particle gun (BioRad, Richmond, CA) as described by Weeks et al. (1993). Plasmid DNA was precipitated onto 1 μm diameter gold particles immediately prior to bombardment and microprojected at 1100 psi pressure in a vacuum of 26 in. (66 cm) Hg into calli formed from embryos cultured for one week. After gene delivery, calli were incubated in the dark at 25 °C for 3 weeks with weekly transfers to fresh recovery media, MS basic medium supplemented with 2.5 g/l of Phytagel and 1 mg/l of 2,4-D.

2.3. Selection of transgenic plants

For positive mannose selection, calli were transferred to selection/regeneration MS modified media containing 5 g/l maltose, 10 g/l mannose, and 0.2 mg/l of 2,4-D. Following growth with 16 h of light and 8 h of darkness at 25 °C for 3 weeks, the regenerated shoots were transferred for rooting induction to Pyrex culture test tubes (25 × 150 mm) containing 18 ml of half-strength MS medium lacking hormones and supplemented with 15 g/l mannose (and no maltose).

Bialaphos selection was performed by moving calli to selection/regeneration medium consisting of MS basic medium supplemented with 0.2 mg/l 2,4-D and 3 mg/l of bialaphos (Meiji Seika Kasha, Tokyo, Japan) and incubating them under the conditions described above for mannose-selected transformants. For rooting induction, the regenerated shoots were transferred to Pyrex culture test tubes

containing 18 ml of half-strength basic medium lacking hormones and supplemented with 3 mg/l of bialaphos.

Plantlets that showed healthy growth under either selection were transferred from rooting media to pots of soil mixture, incubated in a growth chamber for about 2 weeks with decreasing humidity, and transferred to the greenhouse, with supplementary lighting provided by sodium lamps with a day temperature of 17–20 °C and a night temperature of 14–16 °C.

2.4. Chlorophenol-red assay (CPR)

The chlorophenol-red assay (Kramer et al., 1993) was conducted in 96-well plates with each well containing 500 μl of MS maintenance medium adjusted to pH 6.0 and 50 mg/l chlorophenol-red pH indicator, which is a deep red color at this pH. To identify *pmi* transformants, the medium contained 10 mg/l maltose and 15 mg/l mannose; to identify *bar* transformants, the medium contained 3 mg/l of bialaphos. One-centimeter of leaf pieces from regenerating plants after selection or from untransformed control plants were placed in each well. The degree of color change in each well was assessed after 3–4 days incubation in a lighted growth chamber.

2.5. Molecular analysis

Genomic DNA of durum wheat shoots from regenerated plants classified as transformed by the CPR assay and from non-transgenic control shoots was isolated according to Dellaporta et al. (1983). To confirm the presence of *pmi* and *bar* in the putative transformants, fragments were amplified from genomic DNA with the following *pmi* (Syngenta, personal communication) and *bar* (He et al., 1999) specific primers:

PMI-1 = ACAGCCACTCTCCATTCA
PMI-2 = GTTTGCCATCACTTCCAG
BAR-1 = GTCTGCACCATCGTCAACC
BAR-2 = GAAGTCCAGCTGCCAGAAAC

PCR analyses were performed in 25 μl reaction mixtures, each containing 100 ng template DNA, 2 μm of each primer pair, 200 μm of each dNTP, 2.5 mm $MgCl_2$, 10 mm Tris–HCl, pH 8.3, 10 mm KCl, and 1 unit of *Taq* DNA polymerase. Amplifications were conducted in a Perkin Elmer DNA Thermal Cycler with the following protocol: initial denaturation was at 95 °C for 3 min, followed by 35 cycles of [95 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min] and then a final extension at 72 °C for 10 min. The amplification products were resolved on 1.5% agarose gels and stained with ethidium bromide.

3. Results

3.1. Preliminary studies of mannose effects on durum wheat embryos and calli

To establish the best sugar concentrations for the selection of transformed durum wheat plants, preliminary studies were conducted using wheat embryos and callus. Durum wheat embryos (21 days post-anthesis) were excised from caryopses and placed on media containing various concentrations of mannose and/or maltose (Table 1). With maltose as sole carbon source (Treatment I), all the embryos germinated and developed into plants. Without any carbon source (Treatment II), most embryos germinated, but none developed into plants. With mannose as sole carbon source (Treatments III and IV), germination was reduced to 80% (1% mannose) and 77% (2% mannose). The combination of 0.5% of maltose and 1% of mannose gave 90% germination frequency, with 5% of embryos developing into plants.

The ability of calli to grow on mannose and/or maltose was investigated by culturing 1-week-old calli on these carbon sources for 5 weeks. Fresh weights were measured every week (Fig. 1). Calli grown on media with 4% maltose, the standard amount of sugar used for wheat callus culture, doubled in fresh weight within two weeks. No growth was observed in the absence of maltose, even when the media contained 1, 1.5 or 2% mannose. Calli grown without maltose decreased in fresh weight due to the loss of cell

Table 1
Effects of different concentration of mannose and maltose on germination of isolated durum wheat embryos

Experiment	Sugar combination	Concentration in culture medium (%)	Isolated embryos (No.)	Germinated embryos (%)	Plantlet formation (%)
I	Maltose	4	40	100	100
	Mannose	0			
II	Maltose	0	38	98	0
	Mannose	0			
III	Maltose	0	40	80	0
	Mannose	1			
IV	Maltose	0	40	77	0
	Mannose	2			
V	Maltose	0.5	40	90	5
	Mannose	1			

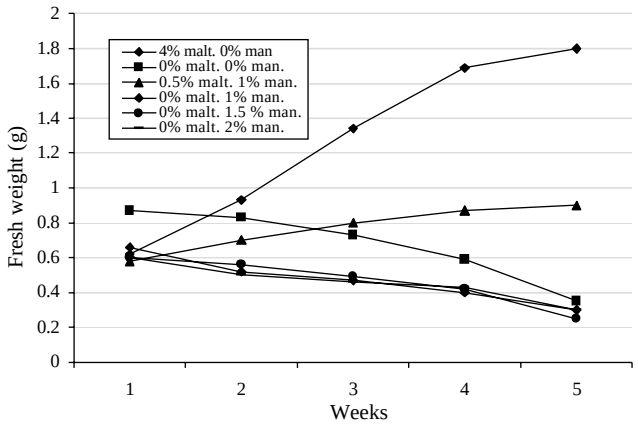

Fig. 1. Mannose dose response curve for durum wheat cv. Svevo calli, 1-week-old, cultured for 5 weeks. Fresh weight was measured every week.

turgidity; the presence of large brown areas after 5 weeks of culture indicated that the calli were dying. The mixture of 0.5% maltose and 1% mannose allowed slow but continuous callus growth. Based on these results, we chose this mixture for positive selection of *pmi*-transformed calli.

3.2. Wheat transformation

Cultivar Svevo was selected for transformation because, among durum wheats, it showed a high percentage of plant regeneration from tissue culture (Gadaleta et al., 2002). Four independent transformation experiments were conducted, two using as DNA the pNOV2820 plasmid containing the *pmi* gene (Miles and Guest, 1984), and two using the pAHC25 plasmid containing the *bar* gene under maize *Ubiquitin1* promoter control (Christensen and Quail, 1996). Five hundred embryos were excised for each experiment, but only embryogenic calli were bombarded.

To increase the survival of transformants, calli were maintained for three weeks after bombardment on a 'recovery' medium without any selection pressure. During this period, cells were able to repair mechanical damage incurred during bombardment. After recovery, calli were transferred to regeneration media that contained either mannose or bialaphos as the selective agent. Plantlets able to regenerate were tested for expression of *pmi* or *bar* genes using the chlorophenol-red (CPR) assay. Leaf pieces, 1 cm long, were incubated on medium supplemented with the CPR pH indicator. The medium is initially pH 6.0 and a deep red color. In the presence of mannose (Fig. 2), the metabolic activity of *pmi*-transformed actively growing cells acidifies the medium and changes its color to yellow. Non-transformed leaves in the presence of mannose do not change the media color. When bialaphos is present in the media non-transformed cells produce ammonia, which raises the pH of the medium and turns the CPR indicator purple (not shown). Leaves from *bar* transformants do not change the color of the medium.

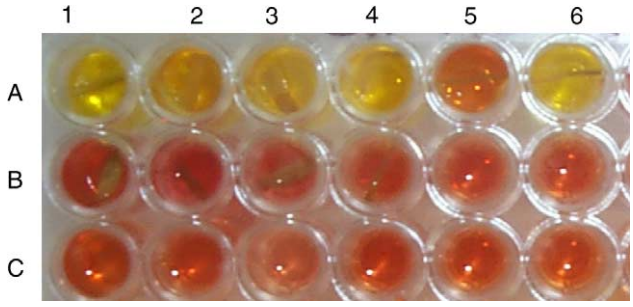

Fig. 2. Chlorophenol-red (CPR) assay of *pmi* transgenic durum wheat plants. Medium surrounding *pmi* transgenic cells is yellow (wells A1, A2, A3, A4 and A6); in contrast, the medium surrounding non-transgenic cells was not acidified and the color remained red (wells A5 and all B wells). C, wells contain leaf pieces from non-transformed plants.

Based on the results of the CPR assay, plantlets that expressed either the *bar* or *pmi* gene were transferred to pots containing a soil mixture and subsequently to the greenhouse. The presence of the transgenes was confirmed by PCR analysis of genomic DNA. A total of 10 mannose-resistant plantlets containing the *pmi* gene and 9 bialaphos-resistant plantlets containing the *bar* gene were obtained. The average biolistic transformation frequencies obtained using the positive and negative selection systems were approximately the same: 1.14% for the PMI-based selection and 0.99% for bialaphos-based selection (Table 2). Stable transformation was confirmed by inheritance of the transgene by PCR analysis of progeny DNA (not shown).

Mannose selection during shoot regeneration was very efficient; in fact, after 4 weeks culture, 27.3% of calli produced green spots that then developed into plantlets. Calli without green spots were cultured for an additional 5 weeks, but only the 1% of them developed into plantlets. Regeneration frequency was significantly higher (42.6%) using bialaphos as selective agent. Selection efficiency, calculated for both methods as the number of T_0 plants containing the selectable marker gene divided by the total number of regenerated plantlets, was significantly higher for mannose selection, 90.0%, than for bialaphos selection, 26.4% (Table 3).

Table 2
Efficiency of durum wheat (cv. Svevo) transformation by the biolistic method using *pmi* or *bar* as selectable genes

Experiment	Gene	Embryogenic calli bombarded (No.)	Transgenic fertile plants (No.)	Transformation efficiency[a] (%)
I	*pmi*	450	6	1.33
II	*pmi*	420	4	0.95
Mean				1.14
III	*bar*	460	4	0.87
IV	*bar*	447	5	1.12
Mean				0.99

[a] Transformation efficiency was calculated as: (No of transgenic plants/No of embryogenic calli bombarded) × 100.

Table 3
Selection efficiency, regeneration, plantlet formation and rooting frequencies in durum wheat transformation experiments employing mannose or bialaphos selection

Selective agent	Regeneration frequency[a] (%)	Plantlet formation frequency[b] (%)	Rooting frequency[c] (%)	Selection efficiency[d] (%)
Mannose	27.3	17.0	50.0	90.1
Bialaphos	42.6	41.5	32.9	26.4

[a] Regeneration frequency = % of calli with at least one green region.
[b] Plantlet formation frequency = (No. of propagated shoots/No. of regenerating calli) × 100.
[c] Rooting frequency = % of shoots developing roots.
[d] Selection efficiency = (No. of transgenic plants/No. of total plants obtained) × 100.

4. Discussion

Our results show that mannose selection of *phosphos-mannose isomerase* transformants is a valid alternative to the use of the herbicide bialaphos in transformation experiments. The PMI enzyme is common in nature and found in all kingdoms, although less so in plants (Reed et al., 2001). The enzyme has been reported to be present in soybean and several other legumes, but absent in many other plants (Goldsworthy and Street, 1965; Lee and Matheson, 1984). It is in the latter group of plants that the *pmi* gene could potentially be used as a selectable marker in transformation experiments. Furthermore, preliminary safety assessment of the *E. coli* PMI protein found no adverse effects with respect to mammalian toxicity, allergenicity, or unintended changes in agronomic properties or nutritional composition of maize and rice transformants (Reed et al., 2001). Transformations with the *pmi* gene were successfully performed in several species including maize (Wright et al., 2001), rice (Datta et al., 2003), sugar beet (Joersbo et al., 1998), hexaploid wheat (Wright et al., 2001) and pearl millet (Kennedy et al., 2004). The results of the present research demonstrated that positive selection using *pmi* as a marker gene in conjunction with mannose is as useful in identifying durum wheat transformants as the most commonly used selectable marker gene, *bar*.

Our application of the CPR assay to leaves in the presence of phosphinothricin or mannose enabled us to accurately identify transgenic plants. All plantlets classified as synthesizing the PMI or PAT enzyme by this assay contained the *pmi* or *bar* gene, respectively. We conclude that the cholorophenol-red assay is a rapid and precise method to identify transformed durum wheat plants.

In the present paper, mean transformation frequency was 1.11% using *pmi* positive selection and 0.99% for *bar* negative selection. Published papers on biolistic transformation of wheat with several genes have reported efficiencies ranging from 1 to 3% (Barro et al., 1998; Blechl and Anderson, 1996; Bliffeld et al., 1999; Ortiz et al., 1996; Witrzens et al., 1998). Recently, transformation efficiency values of 20%, using *pmi* as marker gene, were reported for maize and wheat (Wright et al., 2001). The authors attribute the high efficiency to the absence, in the media, of toxic metabolites produced by non-transformed dying cells, observed as necrotic spots on calli, that may inhibit the regeneration of transformed cells. Dying cells are characteristic of negative selection procedures using antibiotics or herbicides. In our work using mannose selection, necrotic sectors were rarely observed in tissue culture. Rather, the selection appeared to act by preventing non-transformed plantlets from developing roots. In some cases, *pmi* transgenic plantlets showed white basal leaves due perhaps to mannose accumulation in cells and concomitant transcriptional repression of genes associated with photosynthesis and the glyoxylate cycle (Jang and Sheen, 1997).

The significantly lower value of transformation efficiency obtained in our experiments, compared to Wright

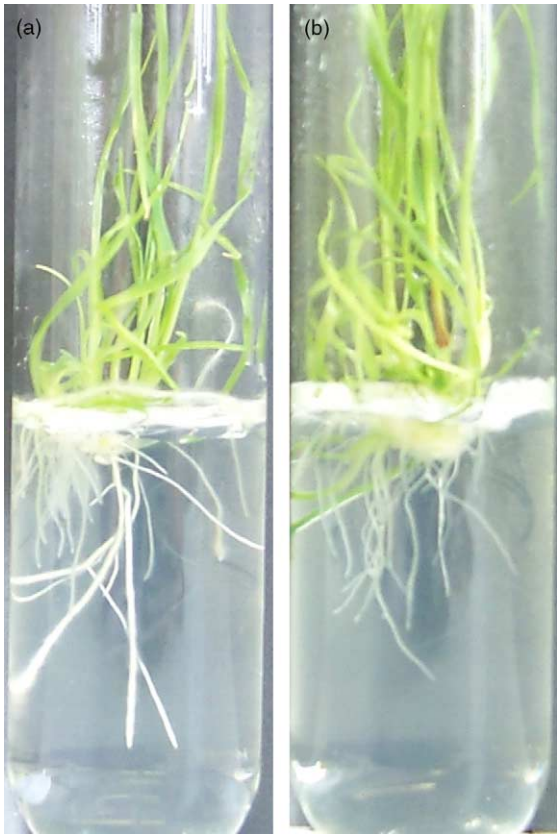

Fig. 3. Root regeneration of transformed durum wheat cv. Svevo plants under negative selection with bialaphos (a) and positive selection with mannose (b).

et al. (2001), could be due to several factors, such as the use of a different genotype or transformation conditions. One important factor could be our continuation of selection during root formation. In fact, Lucca et al. (2001) reported that rice *pmi* transgenic plants cultured on 2% mannose medium during root development died within a few weeks. We observed that transformed wheat plantlets developed roots very slowly on medium containing mannose, and the roots were thin and lacked root-hairs, even after 4–5 weeks of culture (Fig. 3). In contrast, *bar* transgenic plantlets developed multiple roots with root hairs in 2 weeks. We speculate that mannose-selected transgenic plants were lost during root formation. It is at this time that photosynthesis becomes the dominant carbon source for the plantlets, a process that may be inhibited by the presence of mannose.

Although our durum wheat transformation efficiencies were the same using *pmi*/mannose and *bar*/bialaphos, selection efficiencies were much higher (90.1 vs. 26.4%) with the positive selection system. A higher selection efficiency is advantageous in reducing the time and space that are needed to advance cultures and plantlets that are not actually transformed ('escapes'). In addition, use of the *pmi*/mannose system replaces the use of negative selection protocols that rely on resistances to herbicides or antibiotics. We conclude that the positive *pmi*/mannose selection is the best choice for efficient durum wheat transformation experiments and for mitigating concerns about the release of marker genes into the environment.

Acknowledgements

This research project was supported by grants from Università degli Studi di Bari, Italy, project: Fondi Ateneo 2003.

References

Barro, F., Cannell, M.E., Lazzeri, P.A., Barcelo, P., 1998. The influence of auxins on transformation of wheat and analysis of transgene integration patterns of transformants. Theoretical and Applied Genetics 97, 684–695.

Blechl, A.E., Anderson, O.D., 1996. Expression of a novel high-molecular-weight glutenin subunit gene in transgenic wheat. Nature Biotechnology 14, 875–879.

Bliffeld, M., Mundy, J., Potrykus, I., Futterer, J., 1999. Genetic engineering of wheat for increased resistance to powdery mildew disease. Theoretical and Applied Genetics 98, 1079–1086.

Christensen, A.H., Quail, P.H., 1996. Ubiquitin promoter-based vectors for high level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Research 5, 213–218.

Daniell, H., Datta, R., Varma, S., Gray, S., Lee, S-B., 1998. Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nature Biotechnology 16, 345–348.

Datta, K., Baisakh, N., Oliva, N., Torrizo, L., Abrigo, E., Tan, J., Rai, M., Rehana, S., Al-Babili, S., Beyer, P., Potrykus, I., Datta, S.K., 2003. Bioengineered 'golden' indica rice cultivars with beta-carotene metabolism in the endosperm with hygromycin and mannose selection systems. Plant Biotechnology Journal 1, 81–90.

Dellaporta, S.L., Wood, J., Hicks, J.B., 1983. Isolation of DNA from higher plants. Plant Molecular Biology Reporter 4, 19–21.

Erikson, O., Hertzberg, M., Nasholm, T., 2004. A conditional marker gene allowing both positive and negative selection in plants. Nature Biotechnology 22, 455–458.

Gadaleta, A., Blechl, A., Nguyen, S., Blanco, A., 2002. Embryogenic callus induction and plant regeneration variability among Italian durum wheat cultivars. Proceedings of the EUCARPIA Cereal Section Meeting. Salsomaggiore, Italy, 21–25 November 2002, EDS C. Marè P. Faccioli, A.M. Stanca, pp. 440–442.

Goldsworthy, A., Street, H.E., 1965. The carbohydrate nutrition of tomato roots VIII: the mechanism of the inhibition by D-mannose of the respiration of excised roots. Annals of Botany 29, 45–58.

He, G.Y., Rooke, L., Steele, S., Bekes, F., Gras, P., Tatham, A.S., Fido, R., Barcelo, P., Shewry, P.R., Lazzeri, P.A., 1999. Transformation of pasta wheat (*Triticum turgidum* L. var. *durum*) with high-molecular weight glutenin subunit genes and modification of dough functionality. Molecular Breeding 5, 377–386.

Hohn, B., Levy, A.A., Puchta, H., 2001. Elimination of selection markers from transgenic plants. Current Opinion in Biotechnology 12, 139–143.

Jang, J.C., Sheen, J., 1997. Sugar sensing in higher plants. Trends in Plant Science 2, 208–214.

Jauhar, P.P., 2001. Genetic engineering and accelerated plant improvement: opportunities and challenges. Plant Cell, Tissue and Organ Culture 64, 87–91.

Joersbo, M., Donaldson, I., Kreiberg, J., Petersen, S.G., Brunstedt, J., Okkels, F.T., 1998. Analysis of mannose selection used for transformation of sugar beet. Molecular Breeding 4, 11–117.

Kennedy, M.M.O., Burger, J.T., Botha, F.C., 2004. Pearl millet transformation system using the positive selectable marker gene phosphomannose isomerase. Plant Cell Reports 22, 684–690.

Kramer, C., DiMaio, J., Carswell, G.K., Shillito, R.D., 1993. Selection of transformed protoplast-derived *Zea mays* colonies with phosphinothricin and a novel assay using the pH indicator chlorophenol red. Planta 190, 454–458.

Lafayette, P.R., Parrott, W.A., 2001. A non-antibiotic marker for amplification of plant transformation vectors in *E. coli*. Plant Cell Reports 20, 338–342.

Lee, B.T., Matheson, N.K., 1984. Phosphomannoisomerase and phosphoglucoisomerase in seeds of *Cassia coluteoides* and some other legumes that synthesize galactomannan. Phytochemistry 23, 983–987.

Lucca, P., Ye, X., Potrykus, I., 2001. Effective selection and regeneration of transgenic rice plants with mannose as selective agent. Molecular Breeding 7, 43–49.

Miles, J.S., Guest, J.R., 1984. Nucleotide sequence and transcriptional start point of the phosphomannose isomerase gene (manA) of *Escherichia coli*. Gene 32, 41–48.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15, 473–497.

Ortiz, J.P.A., Reggiardo, M.I., Ravizzini, R.A., Altabe, S.G., Cervigni, G.D.L., Spitteler, M.A., Morata, M.M., Elias, F.E., Vallejos, R.H., 1996. Hygromycin resistance as an efficient selectable marker for wheat stable transformation. Plant Cell Reports 15, 877–881.

Ow, D.W., 1996. Recombinase-directed chromosome engineering in plants. Current Opinion in Biotechnology 7, 181–186.

Reed, J., Privalle, L., Powell, L., Meghji, M., Dawson, J., Dunker, E., Suttie, J., Wing, A., Launis, K., Kramer, C., Chang, Y.F., Hansen, G., Wright, M., 2001. Phosphomannose isomerase, an efficient selectable marker for plant transformation. In Vitro Cellular and Development Biology—Plant 37, 127–132.

Weeks, T., 2000. Transformation of wheat with the cyanamide hydratase gene (Patent no. 09/518,988). U.S. Department of Agriculture.

Agricultural Research Service. Available on world wide web: http://ott.arsusda.gov/Inv/a518988.html

Weeks, J.T., Anderson, O.D., Blechl, A.E., 1993. Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*). Plant Physiology 102, 1077–1084.

Witrzens, B., Brettell, R.I.S., Murray, F.R., McElroy, D., Li, Z., Dennis, E.S., 1998. Comparison of three selectable marker genes for transformation of wheat by microprojectile bombardment. Australian Journal of Plant Physiology 25, 39–44.

Wright, M., Dawson, J., Dunder, E., Suttie, J., Reed, J., Kramer, C., Chang, Y., Novitzky, R., Wang, H., Artim-Moore, L., 2001. Efficient biolistic transformation of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) using the phosphomannose isomerase gene, *pmi*, as the selectable marker. Plant Cell Reports 20, 429–436.